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A multicentre validation of Metasin: a molecular assay for the intraoperative assessment of sentinel lymph nodes from breast cancer patients

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A multicentre validation of Metasin: a molecular assay for the intraoperative assessment of sentinel lymph nodes from breast cancer patients

Aims: Treatment strategies for breast cancer continue to evolve. No uniformity exists in the UK for the management of node-positive breast cancer patients. Most centres continue to use conventional histopathology of sampled sentinel lymph nodes (SLNs), which requires delayed axillary clearance in up to 25% of patients. Some use touch imprint cytology or frozen section for intraoperative testing, although both have inherent sensitivity issues. An intraoperative molecular diagnostic approach helps to overcome some of these limitations. The aim of this study was to assess the clinical effectiveness of Metasin, a molecular method for the intraoperative evaluation of SLNs.

Methods and results: RNA from 3296 lymph nodes from 1836 patients undergoing SLN assessment was analysed with Metasin. Alternate slices of tissue were examined in parallel by histology. Cases deemed to be discordant were analysed by protein gel electrophoresis. There was concordance between Metasin and histology in 94.1% of cases, with a sensitivity of 92% [95% confidence interval (CI) 88–94%] and a specificity of 97% (95% CI

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95–97%). Positive and negative predictive values were 88% and 98%, respectively. Over half of the discordant cases (4.4%) were ascribed to tissue allocation bias (TAB).

Conclusions: Clinical validation of the Metasin assay suggests that it is sufficiently sensitive and specific to make it fit for purpose in the intraoperative setting.

Keywords: axillary clearance, breast cancer, CK19, intraoperative, mammaglobin, Metasin, molecular, PCR, sensitivity, sentinel lymph node, specificity

Introduction

Axillary nodal status remains an important prognostic indicator in breast cancer patients.[1,2] The sampling and assessment of sentinel lymph nodes (SLNs) is the accepted standard for nodal staging in breast cancer in the UK, although there is marked divergence in local practice, with most centres (85.6%) carrying out conventional histopathology with a two-stage axillary clearance.[3–5] A minority of centres carry out intraoperative frozen section (FS) (3.8%), touch imprint cytology [DAB cytology (DABc)] (3.1%), or intraoperative molecular testing of SLNs (7.5%).[5] These methodologies have limitations in the context of one-step axillary clearance. Conventional histopathology has the disadvantage of a delayed time course of 2–3 days, and FS and DABc, although highly specific, have inherent sensitivity issues.[6–9] The molecular approach has the disadvantages of cost and disruption resulting from the implementation of a new technology, but it offers the advantages of enhanced sensitivity and specificity.

Detailed histopathological studies of SLNs and axillary lymph nodes resected in unselected patient groups have identified nodal involvement in 25–35% of patients,[10–13] indicating that selective identification of these patients could avoid the consequences of axillary clearance and its complications in the remaining non-involved group. Conventional histological haematoxylin and eosin staining and assessment of SLNs precludes surgical treatment in a one-step process. Preoperative ultrasound-based staging of the axilla with fine needle aspiration cytology (FNAC) or needle biopsy can effectively provide a one-step operative procedure for both breast surgery and axillary clearance, but the radiological axillary staging procedure is inaccurate and prone to overlooking node-positive cases, as it can miss both macrometastases and micrometastases.[12–16]

Examination of DABc preparations and FS diagnosis of SLNs are currently used in clinical practice as a means for rapid assessment of the SLN, facilitating axillary clearance in a one-step process, particularly in the USA.[7] However, these methods have varying degrees of uncertainty in diagnosing nodal involvement.[6,8,9] In the absence of a definitive cost-effective assay, many laboratories in the UK still carry out SLN sampling assessment and subsequent axillary clearance as a two-stage procedure.[5,17] The disadvantages of this approach are considerable: for the patient, not knowing whether there is a need for a second procedure; for the National Health Service (NHS), the cost of the second procedure (£5500 per patient in the UK)[18,19] and the morbidity associated with this process; and for the surgeon, the inherent disadvantages of re-entering a healing surgical wound site, weeks later. In contrast, a single operative procedure is attractive in terms of patient choice, cost, and the avoidance of a second procedure in the 10–20% of node-positive patients (post-axillary sonography).[14–16]

Advances associated with molecular diagnosis have provided tools for the rapid and accurate intraoperative testing of SLNs.[20–24] Although such tools require the implementation of new technologies, they promise the combined benefits of enhanced speed, simplicity, sensitivity, and specificity, thus facilitating intraoperative axillary clearance of lymph nodes as a one-step process in breast cancer patients.[20–24] We have previously described the Metasin assay,[24] which uses a similar methodology to Genesearch (Veridex LLC, NJ, USA), now withdrawn from sale because of the lack of uptake in the USA and commercialization of this product worldwide.[25] Like Genesearch,[20–22] Metasin utilizes real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) to probe for the presence of two predictive markers for the detection of epithelial cells, i.e. cytokeratin 19 (CK19) and mammaglobin (MGB); a third marker, porphobilinogen deaminase (PBGD), provides an internal positive amplification control to confirm the presence of amplifiable RNA. A positive result for either or both of the epithelial cell-specific markers and the positive control suggests the presence of metastatic disease within the tested lymph node. Our previous report[24] described the rationale for the use of these biomarkers

on a cohort of 154 cases, and validated its use on a further 193 samples. We now extend this small study with a larger cohort of breast cancer patients, and describe the clinical validation of Metasin in 1836 cases and report an overall discordance rate of 4.4%.

Materials and methods

CONSENT AND ETHICAL APPROVAL

The study was ethically approved by the Essex 2 Research Ethics Committee (07/H0302/129 & REC 10/H301/24). All patients were recruited after the implementation of the 'New Start' programme.[26,27]

PATIENT SELECTION

Participants were recruited from five centres, with a recruitment target in excess of 1200 cases to ensure determination of statistical significance based on power analysis. Patients subjected to SLN sampling were included in the study if they had biopsy-proven breast cancer and had been previously radiologically staged as negative for axillary disease by a combination of ultrasound and FNAC. Cases that were radiologically deemed to be positive and underwent FNAC were excluded if these were positive, as these patients would undergo axillary node clearance routinely in a one-step procedure. Informed consent was obtained for the intraoperative Genesearch assay analysis and for the prospective intraoperative analysis with Metasin. There were no other exclusion criteria. Standards for Reporting of Diagnostic Accuracy (STARD)-based tabulated detail is provided in Data S1.

There were two groups: an analytical validation group, and a prospective validation group. The recruitment period for the analytical validation group was from March 2008 to August 2010. Homogenates, and/or RNA, were obtained from 448 cases examined with Genesearch. From August 2010 to August 2013, 1388 cases were examined with Metasin for the prospective validation group. Investigators at Princess Alexandra Hospital (PAH), Harlow were blinded to the results of the outcome of Genesearch and histology, and axillary clearance data. The case-based results of the patient demographics, outcomes of Genesearch, histological outcomes of the SLNs and axillary clearances (Table S1) were obtained and collated at PAH.

Analytical validation (Table S1)

Lymph node homogenates and RNA samples ($n = 448$) previously analysed with Genesearch were obtained from four centres that had been using Genesearch: Queen Alexandra Hospital, Portsmouth ($n = 190$); Royal Free Hospital, London ($n = 45$); University Hospital of Wales, Cardiff ($n = 70$);[28] and Prince Philip Hospital, Llanelli, Wales ($n = 143$). Cases from PAH ($n = 154$) were excluded, as these had been previously published in the validation paper.[24] Three centres provided frozen lymph node homogenates of volumes ranging from 1 ml to 5 ml, which were stored at −80°C; the Royal Free Hospital provided RNA, which had also been stored at −80°C. Homogenates and RNA were transported on dry ice to PAH.

Prospective validation (three centres)

Standardization of the assay across the three centres was achieved by using a single source and batch of RT-qPCR primers and probes (TIB Molbiol, Berlin, Germany), ensuring PCR cycling parameters and appropriate machine settings.[24] The outcome of the assay was compared with histology, the gold standard.

Once Metasin had been validated for local use,[24] a further 1388 cases were recruited into the study from three centres (PAH NHS Trust, Harlow, Queen Alexandra Hospital NHS Trust, Portsmouth, and Prince Philip Hospital, Llanelli, Wales). These patients consented to undergo Metasin assay after an in-house validation of the assay in each of the three centres. The outcomes of the results of these 'live' cases were reported to the surgeons intraoperatively.[24] SLNs from each patient were prepared for intraoperative analysis (see below), and the results were reported for surgeons to carry out axillary clearance in positive cases. If positivity for metastatic disease was found, axillary clearance was carried out in a one-step procedure. Metasin assay was repeated at PAH for a representative number of cases (358), to confirm the validity of locally reported results.

ASSESSMENT OF SLNS: LOCALIZATION OF LYMPH NODES AND PREPARATION FOR INTRAOPERATIVE ANALYSIS

The SLNs were identified by use of a combined technique employing radioactively (TcM99m) labelled nanocolloid and 2 ml of diluted Patent V blue dye.[27] Excess extracapsular fat was removed from the lymph nodes, which were weighed and sliced into 2-mm sections, with alternate slices being allocated for homogenization for molecular assessment (Genesearch and/or Metasin) and histopathological assessment. SLNs were serially sliced at standardized 2-mm intervals by consultant pathologists and trained biomedical

scientists (BMSs). No cases were excluded on the basis of size or weight. The numbers of SLNs submitted varied from one to up to six initially, but averaged two to three once the procedure was well established.

HISTOPATHOLOGICAL PROCESSING

Slices for histology were processed individually according to local histopathology protocols. All tissue submitted for histopathology was analysed with conventional methods employing formalin fixation and paraffin embedding. SLNs were analysed at at least three levels in all of the centres. The Royal Free Hospital step-sectioned at 100 μm through the entire tissue block for discordant cases. In discordant cases, slides were examined by two experienced consultant histopathologists during this validation period (the local reporting pathologist and the lead breast pathologist). Discordant node analysis included examination of a further three to five levels with immunostaining for cytokeratins (MNF116). Cases that continued to be discordant despite examination of deeper levels on the SLN were examined with a view to determining the cause of the discordance. These 'discordant' cases were further examined by examination of deeper levels on axillary clearances (selectively) and, where protein lysates were available, by protein gel analysis of homogenates to determine the presence of cytokeratin protein.

HISTOLOGY OF AXILLARY LYMPH NODE CLEARANCE

All centres carried out single-level histological analysis of all dissected lymph nodes. In selected cases during discordant analysis, nodes from the axillary clearance specimens were examined at deeper levels (up to five levels) by cutting step sections at 100 μm through the paraffin wax block to identify the presence of metastatic disease.

RNA PREPARATION AND RT-QPCR ASSAY

RNA extraction of the SLN slices and the RT-qPCR assay were carried out as described previously.[24]

NODAL DISEASE AND PATIENT MANAGEMENT

Macrometastases/micrometastases/isolated tumour cells (ITCs)—histological criteria

Macrometastases were defined histologically as >2 mm, and micrometastases as ≤2 mm and >0.2 mm.[29–31] ITCs were defined histologically as tumour size ≤0.2 mm or a cluster of 200 tumour cells in a single histological section.[29–31]

Macrometastases/micrometastases/ITCs—Metasin criteria

Cases were reported as positive if the RT-qPCR quantification cycle (Cq) values for the molecular analysis were within predetermined 'cut-offs' validated previously.[24,32] Cq values for macrometastases were ≤25 for CK19 and ≤26 for MGB. Cq values for micrometastases were >25 and <32 for CK19, and >26 and <32.3 for MGB. ITCs deemed to be negative were defined by molecular means as >32 for CK19 and >32.3 for MGB. Cq values above these levels were reported as negative.

Axillary lymph node clearances were carried out on all macrometastasis and micrometastasis cases. Local practice changed with time to a more conservative approach for micrometastasis cases, with axillary clearances only for macrometastasis cases in some centres. The different contributing centres followed their local guidelines for axillary clearance based on multidisciplinary team discussions dependent on clinical parameters of stage of disease, size of tumour, histology, and hormone receptor status.

Discordant cases: defined by case-based discordant analysis

Cases were deemed to be discordant when the histology results (positive or negative) did not correlate with the intraoperative molecular analysis results (positive for macrometastasis or micrometastasis; or negative).

DISCORDANT CASE ANALYSIS BY PROTEIN GEL ELECTROPHORESIS OF HOMOGENATES FOR CK19

Protein concentrations of homogenates were assessed with a NanoDrop (Thermo Scientific, DE, USA). Protein levels were adjusted to a final concentration of 2 μg/μl in loading buffer (1 × Laemmli sample buffer; BioRad, Hemel Hemstead, UK) containing 5% β-mercaptoethanol (Sigma-Aldrich, Dorset, UK).

Protein samples were boiled for 5 min at 95°C and cooled on ice, and 25 μl of each sample was loaded (50 μg of total protein) onto a 12% sodium dodecylsulphate polyacrylamide gel electrophoresis gel. Following electrophoresis, proteins were electrotransferred onto poly(vinylidene difluoride) (PVDF) membranes (Roche Diagnostics, Burgess Hill, UK), which were then blocked [4% w/v Marvel in Tris-buffered saline containing 1% Tween -20 (TBS-T)] for 1 h at room temperature. PVDF membranes were incubated

with the primary antibodies mouse monoclonal antibody raised against CK19 (A53-B/A2; 1:1000; Abcam, Cambridge, UK) and rabbit anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:1000; Sigma-Aldrich) in blocking solution overnight at room temperature. On the following day, PVDF membranes were washed, and incubated in secondary peroxidase-labelled anti-species IgG (1:5000; Vector Laboratories, Peterborough, UK) in blocking solution for 2 h. After washing with TBS-T, the ECL plus western blotting detection system (GE Healthcare Life Sciences, Buckinghamshire, UK) was used to visualize CK19 and GAPDH antibody complexes, with the Chemidoc-MP imaging system and IMAGE LAB 4.1 software (BioRad, Hemel Hemstead, UK). GAPDH was used as an internal control to confirm equal sample loading.

STATISTICAL ANALYSIS

The data were separated into analytical validation (with Genesearch cases) and prospective validation (without Genesearch cases) case cohorts. The endpoint of the main analysis was the overall agreement between histology, Metasin, and Genesearch, where applicable. This endpoint was assessed after, where possible, the detailed examination of the SLN at deeper levels and immunohistochemistry had been performed. Other aspects of statistical analyses were time for duration of assay (from receipt of node in the lab to reporting result to surgical team) and sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of Metasin. Receiver operator curve (ROC) analysis of datasets was used to confirm Cq values set for macrometastasis and micrometastasis determination.

McNemar's test for significance was used to test the null hypothesis that there is no difference in SLN outcome (positive, including macrometastasis and micrometastasis cases; or negative, including ITC and negative cases) between histology, Metasin, and Genesearch. Spearman's correlation test was also used to assess the degree of correlation between the three groups.

PATIENT MANAGEMENT DURING THE COURSE OF THE VALIDATION STUDY

All cases with confirmed breast cancer were assessed with axillary sonography and FNAC. Outcomes were discussed at multidisciplinary team meetings. Patients with negative FNAC results were offered a one-step procedure with axillary clearance, if positive, following intraoperative assessment with Metasin. The risk of intraoperative assay failure was mitigated by ensuring that alternate slices of the lymph nodes were retained for conventional histopathological analysis. If the assay failed in the intraoperative setting, the assay was repeated. If, however, the assay failed again, surgeons were asked to await conventional histopathological assessment.

Results

PATIENT DEMOGRAPHICS

Details of the patient demographics and relevant clinical information were retrospectively collated, and are presented in Data S1.

RESULTS OF THE CUT-OFF VALUES FOR POSITIVE AND NEGATIVE NODES, MACROMETASTASES, AND MICROMETASTASES

Once all concordant positive cases (between Genesearch and histology) had been identified, the nodes were grouped separately into macrometastasis or micrometastasis cases on the basis of the histology results and Cq data, as described previously.[24,32]

Details of the Metasin and histology data for the analytical validation group with the Genesearch assay and Metasin are provided in Table S1.

STATISTICAL ANALYSIS

Analytical validation cases—correlation of histology, Metasin, and Genesearch

Within the analytical validation group comparing histology with Metasin and Genesearch, there were significant correlations of positive and negative outcomes between Metasin and Genesearch ($P = 0.015$), between histology and Metasin ($P < 0.0001$), and between histology and Genesearch ($P < 0.0001$), with McNemar's test. Spearman's correlation test also showed statistically significant correlations, with P-values of <0.0001 for all three groups.

Prospective validation cases—correlation of histology and Metasin

The same analysis was applied to all cases with histology and Metasin results, including the cases after Genesearch was withdrawn. There was a significant correlation between the two methods (Spearman's correlation, $P < 0.0001$). No statistically significant difference was found between the outcomes of histology and Metasin. This indicates that Metasin gives a

very similar outcome to the existing gold standard of histological assessment.

ROC curve analysis of Cq values for macrometastasis/micrometastasis determination

Cq values for the Metasin markers CK19 and MGB were computed against histology by use of ROC analysis for a small subset of cases (results not shown). The sensitivity, specificity and overall accuracy with 95% confidence intervals (CIs) were obtained, and cut-off points were determined by maximizing the sensitivity and specificity. The cut-off points in this analysis for Metasin (CK19 and MGB) for macrometastasis and micrometastasis was 0.4 from the Cq values determined previously. The MGB macrometastasis cut-off value was 27.9 as compared with 26. The overall accuracy and 95% CIs for Metasin (CK19 and MGB) based on ROC curve analyses were as follows. For CK19, the macrometastasis cut-off was <25.1, with an accuracy of 0.9597 (95% CI 0.943–0.977), and the micrometastasis cut-off was <31.7, with an accuracy of 0.783 (95% CI 0.722–0.844). For MGB, the macrometastasis cut-off was <27.9, with an accuracy of 0.8754 (95% CI 0.843–0.908), and the micrometastasis cut-off was <32.7, with an accuracy of 0.6662 (95% CI 0.58–0.75).

METASIN POOLED PROSPECTIVE AND VALIDATION GROUP OF 1836 CASES: HISTOLOGY VERSUS METASIN

Comparison of Metasin with histology

In total, 1836 cases were tested with Metasin in the combined prospective and analytical validation groups. The results as compared with histology are shown in Table 1 and Figure 1. Of these, 356 cases (19.4%) were both Metasin-positive and histology-positive, and 1372 cases (74.7%) were negative with both. Thirty-two cases (1.7%) were discordant, being histology-positive and Metasin-negative. Conversely, 49 cases (2.7%) were histology-negative but Metasin-positive. The overall discordance rate was 4.4% (Table 1; 32 + 49 = 81 of 1836 cases).

Analysis of lymph nodes in axillary clearance specimen

As Metasin is a predictor of axillary node involvement, we examined the nodes removed at axillary clearance for histological evidence of involvement of selected false-positive cases ($n = 10$). Four cases were shown to have positive histology in the axillary clearance samples (three macrometastasis cases and one micrometastasis case).

SENSITIVITY OF THE MOLECULAR ASSAY

Genesearch as compared with histology

Once discordance analysis of cases had been carried out, the sensitivity of Genesearch as compared with histology was 98% (95% CI 92–99%), and the specificity was 93% (95% CI 89–95%). Genesearch had a PPV of 77% and an NPV of 99%. The overall accuracy was 94%. The discordance rate of Genesearch as compared with histology was 6% (Table S1).

Metasin as compared with histology (all cases, $n = 1836$)

The sensitivity of Metasin as compared with histology was 92% (95% CI 89–94%), and the specificity was 97% (95% CI 95–97%). Metasin had a PPV of 88% and an NPV of 98%. The overall accuracy was 96%. The discordance rate of Metasin as compared with histology was 4.4%.

MACROMETASTASIS/MICROMETASTASIS PREDICTION OF METASIN AS COMPARED WITH HISTOLOGY

The results for the cohort of 356 cases are summarized in Table 2. For the dataset of 356 cases that were histology-positive and Metasin-positive from both the analytical and prospective validation datasets (total, $n = 1836$), tumour volume data (macrometastasis/micrometastasis) were available for

Table 1. Discordant results between histology and Metasin after pooling of retrospective and prospective cases (1836 cases representing 3297 nodes)

	Discordance						
Cases	True positive, no. (%)	True negative, no. (%)	False negative, no. (%)	False positive, no. (%)	Fails, no. (%)	Total, no. (%)	Total discordant cases, no. (%)
After deeper levels on sentinel node	356.0 (19.4)	1372 (74.7)	32 (1.7)	49 (2.7)	27 (1.5)	1836 (100)	81 (4.4)

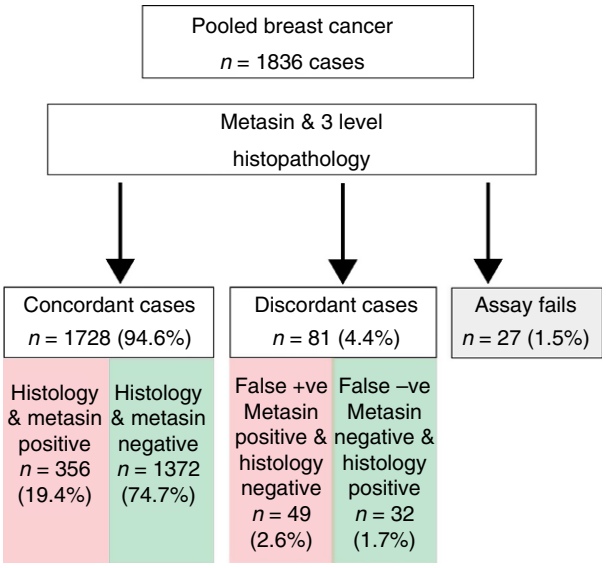

Figure 1. Summary of clinical outcomes for pooled dataset of analytical validation cases and clinical validation cases undergoing analysis with Metasin (n = 1836).

356 cases (Table 3). In two of the positive cases, there was macrometastasis/micrometastasis discordance between two different nodes, and these have been included because they are overall case-concordant. Concordance of macrometastasis/micrometastasis prediction was seen in 88.8% (277 + 39 = 316). Prediction of macrometastasis was seen in 277 of 356 cases (77.8%), and prediction of micrometastasis in 39 of 356 cases (10.9%). There was discordance between macrometastasis and micrometastasis prediction of 11.2% (28 + 12 = 40).

PREDICTIVE MARKERS

Table 3 summarizes the case-based data for marker positivity for all 405 Metasin-positive cases (inclusive of the 49 Metasin-positive histology-negative discordant cases). Of these, 224 cases (210 macrometastasis + 14 micrometastasis = 224, 55.3%) were positive for both CK19 and MGB, 151 cases (102 + 49 = 151, 37%) were positive for CK19, and 30 (11 + 19 = 30, 7.4%) were positive for MGB only. Of the 30 cases positive for MGB only, 11 were macrometastasis cases and 19 were micrometastasis cases.

Table 3. Marker distribution in macrometastasis and micrometastasis cases; the table summarizes all of the Metasin-positive cases (inclusive of Metasin-positive histology-negative discordant cases) deemed to be macrometastases and micrometastases, and the associated marker distribution

Marker	Macrometastases	Micrometastases	Total (%)
CK19 and MGB	210	14	224 (55.3)
CK19	102	49	151 (37.3)
MGB	11	19	30 (7.4)
Total	323	82	405 (100)

CK19, cytokerain 19; MGB, mammaglobin.

PROTEIN GEL ANALYSIS OF HOMOGENATES FROM DISCORDANT CASES

Nylon PVDF membranes prepared from protein gels were probed for the presence of CK19 marker (Table 4; Figure 2): 24 of the 81 discordant cases (29.6%) were macrometastasis cases, and 57 of 81 were micrometastasis cases (70.4%). Homogenates were available for 66 of 81 (81%) cases that were discordant in the study group. Of the 66 informative cases, 22 were macrometastasis cases, and 44 were micrometastasis cases.

Histology-negative Metasin-positive cases (false-positive cases

Protein lysates were available for 38 of 49 cases: 17 were macrometastasis cases, and 21 were micrometastasis cases. Cytokeratin-related bands were seen in 13 of the 17 macrometastasis cases and in 10 of the 21 micrometastasis cases. No cytokeratin-related bands were seen in the remainder of the cases (Table 4; Figure 2).

Table 2. Comparison of tumour volumes in concordant positive cases between histology and Metasin (n = 356)

	Histology			
		Macrometastases, no. (%)	Micrometastases, no. (%)	Total, no. (%)
Metasin	Macrometastases	277 (77.9)	28 (7.8)	305 (85.7)
	Micrometastases	12 (3.4)	39 (10.9)	51 (14.3)
	Total	289 (81.2)	67 (18.8)	356 (100)

Table 4. Discordance analysis by protein gel electrophoresis (see Figure 2); the table summarizes all of the Metasin and histology discordant cases and outcome of the western blots of lymph node homogenates after protein gel electrophoresis and probing for cytokeratin (CK19) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Outcomes are tissue allocation bias (TAB), absence of CK19 protein or that Metasin did not detect CK19 & MGB RNA in the assay.

	'False positive' (histology-negative/Metasin-positive)				'False negative' (histology-positive/Metasin-negative)			
Total no.	49				32			
Homogenates for western blots, no. (%)	38 (58)				28 (42)			
	Macrometastases		Micrometastases		Macrometastases		Micrometastases	
No. of cases (% of 66)	17 (26)		21 (32)		5 (8)		23 (35)	
Outcome of western blots for CK19	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
No of cases (% of 66)	13 (20)	4 (6)	10 (15)	11 (17)	4 (6)	1 (2)	9 (14)	14 (21)
Positive clearances/total clearances	0/9	3/4	6/7	0/3	0/4	0	1/4	1/6
	TAB	No CK19 protein	TAB	No CK19 protein	Assay missed diagnosis	TAB	Assay missed diagnosis	TAB

TAB, tissue allocation bias.

Histology-positive Metasin-negative cases (false-negative cases)

Protein lysates were available for 28 of 32 cases: five were macrometastasis cases, and 23 were micrometastasis cases. Cytokeratin-related bands were seen in four of five macrometastasis cases, and in nine of 23 micrometastasis cases. No cytokeratin-related bands were seen in the remainder of the cases (Table 4; Figure 2).

ASSAY FAILURES: ANALYTICAL AND VALIDATION GROUP

Twenty-seven cases were deemed to be inconclusive (27/1836, 1.5%), of which 20 were Metasin assay failures. Seventeen of the 20 cases (0.9%) reported as assay failures from the archived samples were mainly attributable to the internal reference gene *PBGD* not reaching the set Cq value, implying insufficient or degraded RNA in the material submitted for molecular analysis. Of the remainder, many of these assay failures were seen when insufficient tissue was provided or when fat was not adequately trimmed from material submitted for molecular analysis. Histological review of sections from selected assay failure nodes showed that >50% of the node was replaced by fat, in addition to <50 mg of nodal material being submitted. The remaining three cases in the 'invalid' group were reported as positive on histological analysis of parallel sections (two macrometastasis cases; one micrometastasis case), suggesting possible tissue selection bias. Seven other cases did not yield sufficient tissue for histology. Two of these cases were Metasin-positive (one micrometastasis case; one macrometastasis case), and five were Metasin-negative. As these cases were not corroborated by histology, these cases were excluded from the main study group, and included in the invalid group.

Discussion

This validation study of >1800 SNLs assessed with Metasin with parallel histology shows that the assay is fit for purpose, with an overall discordance rate of 4.4%. This compares favourably with the discordance rates published for Genesearch, an assay that is comparable to Metasin, with discordance rates of 9.2% and 7.7%.[20–22] Our data show that Metasin is at least comparable to conventional histology, the current gold standard. The molecular approach provides a significant major advantage of speed over conventional histology, enabling its use in the intraoperative setting.[21–24]

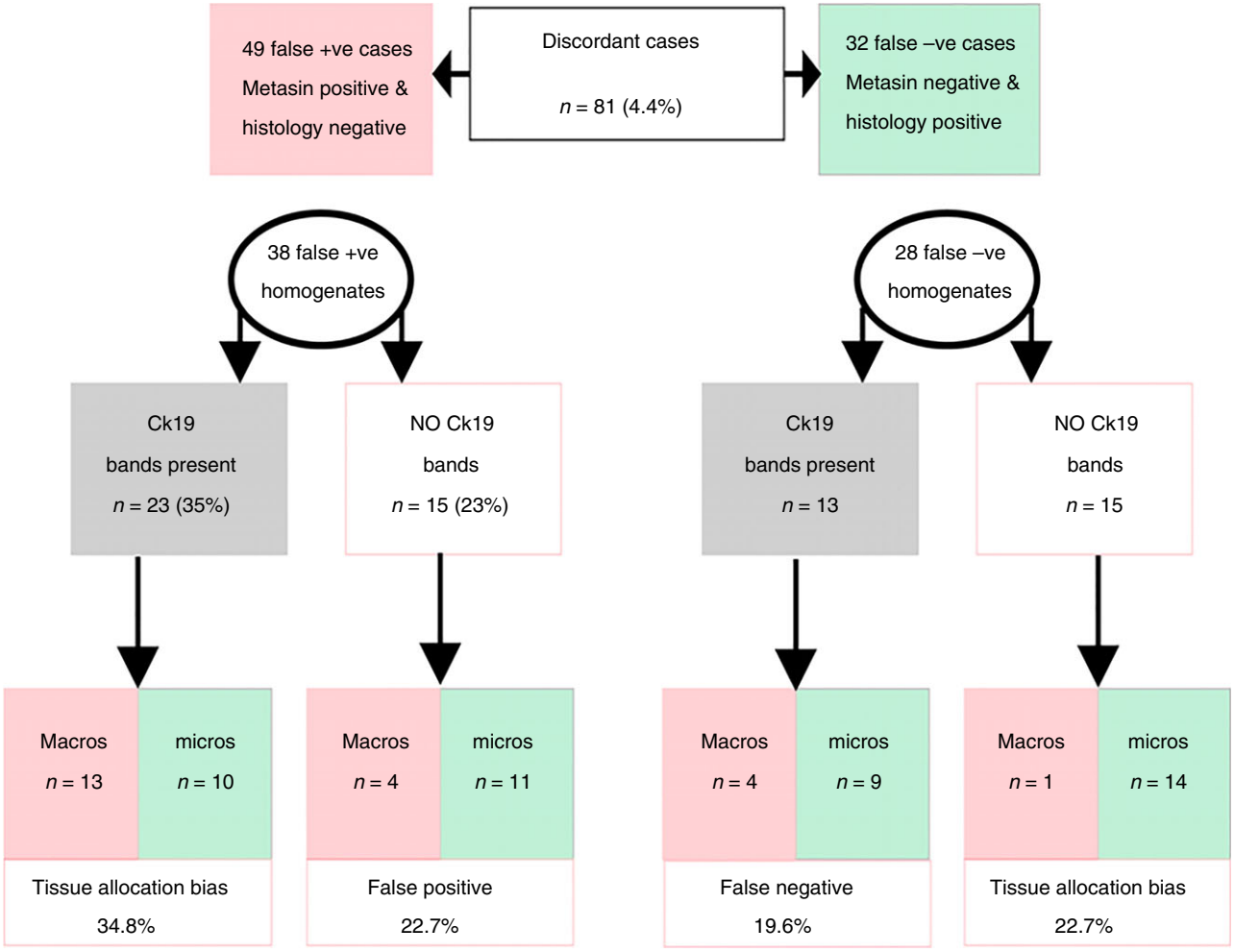

Figure 2. Discordance analysis by protein gel electrophoresis (see Table 4).

Overall, Metasin has a sensitivity of 92% and a specificity of 97%, with an NPV of 98% and a PPV of 88%. These figures compare favourably with those for the cases examined with Genesearch in this study (retrospective samples) and in published data.[20–22] This contrasts with established methods used in the intraoperative setting: FS and DAB cytology.[6–9] A meta-analysis of the published literature has indicated that FS analysis[8] has a sensitivity of 76% and that imprint cytology[6] has a sensitivity of 62%, with marked divergence in the range of sensitivities and specificities reported. A report on intraoperative FSs examined by step sectioning and rapid immunostaining of sections at 50 μm has demonstrated an NPV value of 94%.[11] However, implementation of this approach is impractical in the UK district general hospital diagnostic setting.

Currently, FS and DABc are alternative approaches to Metasin. Although they are cheaper than Metasin,[18,19,33] both have significantly lower sensitivity rates than the molecular approach. Unlike Metasin, which is entirely carried out by trained BMSs, these two methods are dependent on the availability of trained histopathologists to read and report the slides (s). In some NHS Trusts, surgical theatres are located at sites remote from the laboratory, with no immediate access to consultant pathologists. In the UK, the reluctance to embrace intraoperative testing has been largely related to the cost of implementation of the assays, capital costs, and the cost per test[33] of commercially available assays. In addition, there has been a tendency for pathologists to adopt a very conservative stance, and to be reluctant to embrace this alternative approach. However, the driving force for embracing this approach rests with the cost to the NHS of the second operations[19] (£5500) and patient choice, which is largely ignored by histopathologists.

In implementing Metasin, we have elected to submit parallel sections for histology. Discordance between histology and molecular analysis is inevitable. Here, we have investigated our discordant cases by detailed western blot analysis, and have demonstrated that >50% of these discordant cases can be ascribed to tissue allocation bias. Twenty-three per cent of the discordant group classified as false-positive cases (histology-negative; Metasin-positive) did not show CK19 bands on western blot analysis (four macrometastasis cases; 11 micrometastasis cases). These 15 cases were subjected to axillary clearance. In three of four patients from this group with macrometastases, the axillary clearance specimens showed positive nodes, implying that the 'Metasin-positive' call was correct, and that the failure to detect the metastasis by histology was probably attributable to tissue allocation bias. The failure to detect cytokeratin bands in this material is not surprising, because some of these protein lysates were nearly 6 years old. This probably accounts for absence of cytokeratin bands on western blotting in these samples.

The false-negative group (20% of the discordant group) implies that, despite using two predictive markers, we are missing a small number of CK19/MGB-negative cases. Fortunately, these were detected by parallel histology. Recent refinements to the assay with Conformité Européenne (CE) marked Metasin reagents and refinements of polymerase chain reaction conditions have resulted in the ability to detect nearly half of these false-negative cases.

In establishing Metasin, we have mirrored the Genesearch strategy by using more than one epithelial marker, and we acknowledge that, even with the use of two markers, there is a likelihood of missing cases positive for tumour. We and others have realized the shortcomings of using a single marker such as CK19, as is used in some commercial assay (s).[24,34–39] It is therefore significant that a number of cases (7.4%, 30 of 405 Metasin-positive cases) were CK19-negative but MGB-positive. Had we used CK19 only, like the other commercial assay that is available,[23] and subjected the whole node to the assay,[23,36] we are likely to have failed to detect these 30 cases positive only for MGB and the false-negative cases by Metasin or by histology.

Here, we have shown that Metasin is accurately able to detect micrometastasis and macrometastasis in nearly 89% of cases. Discordances between Metasin and histology are inevitable if the strategy of using alternate sections for molecular and histological examination is implemented. The 11% discordance is a drawback, and should be recognized as a disadvantage of adopting the approach of examining parallel sections by conventional histology. Improvements in axillary sonography have increased the rate of preoperative detection of metastatic disease in the axilla, and have hence reduced the numbers of positive intraoperative SLN cases.[14–16] The numbers in the discordant group (owing to small tumours) ascribed to tissue allocation bias and assay misses are likely to continue. This is despite the overall reduction in positive axillas undergoing SLN sampling regardless of the improvements in axillary sonography.[14–16]

Assay failures are inevitable, and are no different from the indecision of pathologists examining FSs who inform the surgeon to await paraffin sections. Overall assay failures were of the order of 1.5% (inclusive of analytical and prospective validation groups), which is not surprising, as the archived homogenates and RNA were 4–6 years old. RNA degradation was evident in this historical group by the failure of *PBGD* amplification, accounting for the high failure rate in this 'archival group', contrasting with 0.7% of assay failures in the prospective validation group. Very small lymph nodes (<50 mg) and lymph nodes that are largely replaced by fat contribute to assay failure, owing to insufficient amplification of *PBGD*, similarly to what occurs with Genesearch. For these reasons, we have assiduously examined parallel sections of tissue for Metasin and histology, and continue to advocate the use of parallel sections for histopathological examination.

The rationale for intraoperative testing and axillary clearance has been recently challenged by the findings of the Z0011 study,[40] advocating a conservative approach to axillary clearance, favouring radiotherapy and or systemic chemotherapy. Currently in the UK, there are two schools of thought: some follow the outcome of the Z0011 study[40] with a conservative approach, and others do not, with some centres adopting a more interventional stance, performing axillary clearances for micrometastases. Prospective studies are underway to evaluate the role of axillary clearance in SLN-positive cases.

Conclusion

The treatment of patients with breast cancer is continually evolving. There has been a significant change in breast cancer management worldwide, with the emphasis drifting towards a less radical approach to surgical treatment over the last 10–15 years. However, current clinical practice still requires the assessment of SLNs.[41,42] The large data-

set described in this article confirms the high sensitivity and specificity of Metasin, and a low level of discordance with histopathology. The ability to diagnose metastatic disease with considerable sensitivity enables the surgeon to consider axillary clearance as a one-step procedure. These advantages, together with patient choice and NHS savings, make a compelling argument for the adoption of this approach.

Conflicts of interest

The Metasin assay was developed within the NHS with funding from local charities and from the Epping Breast Research Fund. The assay in the format described here was prepared with reagents prepared as by Al Ramadhani *et al.* (2013). The authors do not have any conflicts of interest. The intellectual property rights for the Metasin assay rest with PAH NHS Trust, Harlow, Essex. The MGB gene is under patent (US 5668267 A; University of Washington; http://www.google.com/patents/US5668267).

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Author contributions

P. Sai-Giridhar, S. Al-Ramadhani, and D. George: developed the Metasin assay and subsequently refined it. D. George and P. Gopinath: data analysis and preparation of data for figures. P. Gopinath: carried out clinical data collection, data analysis, basic statistical analysis, and preparation of STARD criterion information. S. Jader, A. Findlay, S. Brown, E. Arkoumani, S. Al-Sam, J. G. McKenzie, H. Bradpiece, S. Jenkins, and A. Aylwin: lead pathologists, radiologist, BMS and surgical team members at PAH who carried out surgery and subsequent pathological analysis of lymph nodes. S. Holt and Y. Sharaiha: lead surgical team members at Prince Philip Hospital, Llanelli, UK who carried out surgery and subsequent pathological analysis of lymph nodes. C. Yiangou, A. Agrawal, A. McDowell, G. Gabriel Francis, M. Jeffrey, N. Agrawal, and Ian A. Cree: lead, and surgical team members, pathologists and BMS at Queen Alexandra Hospital, Portsmouth who carried out surgery and subsequent pathological analysis of lymph nodes. R. E. Mansel: lead surgeon at Cardiff University, UK who carried out surgery. M. Keshtgar, N. McDermott, and S. El Sheikh: lead, and surgical team members and pathologists at Royal Free Hospital who carried out surgery and subsequent pathological analysis of lymph nodes. D. Wellsted J. Collard, and H. Chaplin: lead and team members for statistical analysis of data, including ROC curve analysis. W. Andrews: immunoblotting. O. Landt: provided Metasin reagents and helped to develop the assay. S. Bustin and V. Sundaresan: Co-MD supervisors of S. Al-Ramadhani, and proofread manuscripts. M. Sundaresan and V. Sundaresan: pathologists—proofread all drafts of manuscripts.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data. S1. STARD Checklist information.

Table S1. Discordant analysis and case-based outcome in the retrospective group (n = 448): outcomes for histology, Metasin, and Genesearch.